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Heat Shock Activation of the *groESL* Operon of *Agrobacterium tumefaciens* and the Regulatory Roles of the Inverted Repeat

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Deletions were constructed in the conserved inverted repeat (IR) found in the *groESL* operon of *Agrobacterium tumefaciens* and in many other *groE* and *dnaK* operons and genes in eubacteria. These deletions affected the level of expression of the operon and the magnitude of its heat shock activation. The IR seems to operate at the DNA level, probably as an operator site that binds a repressor under non-heat shock conditions. The IR was also found to function at the mRNA level, since under non-heat shock conditions transcripts containing deletions of one side of the IR had longer half-lives than did transcripts containing the wild-type IR. Under heat shock conditions, the half-life of the mRNA was unaffected by this deletion because of heat shock-dependent cleavage. However, the *groESL* operon was found to be heat shock activated even after most of the IR was deleted. This observation, together with the fact that the *groESL* operon of *A. tumefaciens* was heat shock activated in *Escherichia coli* and vice versa, suggests that a heat shock promoter regulates the heat shock activation of this operon. The primary role of the IR appears to be in reducing the MRNA levels from this promoter under non-heat shock conditions.

The heat shock response is a widespread phenomenon found in all living cells examined (10). It is characterized by the induction of several proteins, some of which are highly conserved in evolution, especially those encoded by the *groEL* (*hsp60*) and the *dnaK* (*hsp70*) genes (4, 16, 17, 48).

One of the recent findings concerning the *groE* and *dnaK* operons of many eubacteria is the existence of an inverted repeat (IR) (TTAGCACTC-N_9-GAGTGCTAA) located in the upstream regulatory region. This IR has been found in a large number of phylogenetically distant bacteria (1, 2, 5, 9, 13, 20, 21, 25, 27, 28, 30, 33, 34, 39, 41, 42) and is highly conserved. So far, this IR has been found only in the upstream regions of *groE* and *dnaK* operons or genes, except for two cases in which it is located upstream of the *dnaJ* gene (14, 39). In these two cases, the *dnaJ* gene is separated from the *dnaK* gene, while generally they are organized in the same operon.

The role of the IR in heat shock induction was characterized in two low-GC gram-positive bacteria, *Lactococcus lactis* (39) and *Bacillus subtilis* (46, 49). In the *dnaJ* gene of *L. lactis*, deletion of the IR resulted in the loss of heat shock activation of this gene. In addition, the transcription level at low temperatures was higher without the IR, in comparison to that of the wild-type gene. In the *dnaK* operon of *B. subtilis*, site-directed mutagenesis of the IR resulted in a high level of transcription at low temperatures and a reduction in heat shock activation. In *B. subtilis*, the protein encoded by *orf39* (the first gene in the *dnaK* operon of *B. subtilis*) was found to serve as the repressor and to bind the IR at the DNA level (45). In the *groE* operon of *B. subtilis*, the IR was also shown to function as an operator site and to be involved in determining the half-life of the transcript to which it is connected (46). Under non-heat shock conditions, deletion of the IR results in a longer half-life for the transcript.

In *E. coli*, the heat shock response is mediated by the positive regulator protein sigma-32. This sigma factor recognizes a different promoter sequence from that of the vegetative sigma factor (sigma-70) and in this way specifically transcribes heat shock genes (6, 47). Lately, homologs of *E. coli* sigma-32 were cloned and sequenced from *Agrobacterium tumefaciens*, *Caulobacter crescentus*, and *Zymomonas mobilis*, all of which are alpha purple proteobacteria (26). These sigma factors differ from *E. coli* sigma-32 in several aspects concerning its regulation (26).

In previous papers, we have shown that the *groESL* operon of *A. tumefaciens* contains the highly conserved IR and that the operon is heat shock activated (34). In contrast, the *dnaKJ* operon is heat shock activated but it does not contain this conserved IR (36). Our results indicated the existence of a common, putative, heat shock promoter, and we proposed that both operons are recognized by an alternative sigma factor that is responsible for their heat shock activation.

In this study, we investigated the involvement of the conserved IR in the heat shock response of the *groESL* operon of *A. tumefaciens*. The experiments presented dealt with deletions constructed within the IR. These manipulations of the IR changed (decreased or increased) the level of the mRNA of this operon before heat shock. Deletions within the IR region also reduced the magnitude of heat shock activation of this operon, but heat shock was still observed even after most of the IR was deleted. The deletion of one side of the IR was found to affect the half-life of the transcript by increasing the half-life of the mRNA under non-heat shock conditions in comparison to the mRNA containing the wild-type IR. No effect on the mRNA half-life was found under conditions of heat shock.

The *groESL* operon of *A. tumefaciens* was found to be heat shock activated in *E. coli*, but the mRNA level of this operon was very low, indicating that it wasn't efficiently recognized in *E. coli*. However, the *E. coli groESL* operon was also heat

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TABLE 1. Primers used in this study

Primer	Sequence[a]	Sequence positions[b]
IRF1	**T**CCATCACGGGGACCTTCAA	436–455
IRR1	**T**CCAGCAGAGCCGAGATAAG	411–392
IRR2	**T**CCTTGTTGGAGTGCCAGCA	424–405
UPR1	TTCCACGCGCTGACGAGGTG	292–311
UPF2	TT**C**TTCCGGCAGCGCGTTCT	352–371
UPF3	TTCTTGACTCCGGGAGAGCG	368–387
UPF4	T**T**CTTATCTCGGCTCTGCTG	390–409
PEA1	TGCCTAATCCCTCGATC	491–475
PEC1	CTTCTTTACGCTTGACG	182–166

[a] Sequences are presented from 5′ to 3′. Bases that were changed are in boldface.

[b] Sequence positions in the *A. tumefaciens* sequence are taken from GenBank accession no. X68263; sequence positions in the *E. coli* sequence (the last primer) are taken from reference 18.

shock activated in *A. tumefaciens*, suggesting that the region needed for heat shock activation of the *groESL* operon of *A. tumefaciens* consists solely of the promoter region.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *A. tumefaciens* C58 (ATCC 33970) was used for the preparation of RNA and was cultivated as previously described (34). *E. coli* MC1022 [*araD139* Δ(*ara leu*) *7697* Δ(*lacZ*) M15 *galU galK strA*] (7) was used for transformations. *E. coli* 71-18 [F′ *lacI*^{q} Δ(*lacZ*)M15, *proAB*/Δ*lac-proAB thi supE*] (44) was used for the preparation of RNA and for M13 manipulations. *E. coli* SM-10 (*thi thr leu* su_{III}) (38) was used for conjugations. Heat shock was achieved by transferring exponentially growing cultures of *A. tumefaciens* or *E. coli* cells from 25 or 30 to 42°C, respectively. Plasmid pGS-SV-1 (35) was used as a shuttle vector between *E. coli* and *A. tumefaciens.* The cloning vector pUC18 (44) was used for subcloning.

RNA and DNA manipulations. RNA was prepared from 50-ml cultures of *A. tumefaciens* or from 30-ml cultures of *E. coli* and manipulated as described previously (34, 35). Each lane in Northern (RNA) hybridizations and in primer extension analyses contained 10 μg of RNA, unless otherwise stated. The levels of activation were determined by Northern hybridizations or primer extension and calculated by using a Fuji BAS1000 PhosphorImager. Two of the shuttle vector genes were used as internal controls for mRNA levels. Plasmid DNA for PCR was prepared by using the boiling method (31).

Probes and primers. The *groES* probe used for hybridization with the *A. tumefaciens groESL* operon was a 306-bp *Bst*EII-*Bss*HII fragment (positions 332 to 637; GenBank accession no. X68263) constructed from plasmid pGS-AG-3a (34). The oligonucleotides used for deletion construction and primer extension are listed in Table 1.

Deletion mutagenesis by PCR. Five deletions were constructed by PCR as described by Imai et al. (19). The 1-kb *Eco*RI-*Sal*I fragment of pGS-AG-22 (35) was subcloned into pUC18 digested with the same enzymes, resulting in the plasmid, pGS-AG-16, that was used for PCR mutagenesis. The PCR conditions were 30 cycles at 94°C for 1 min, 60°C for 0.5 min, and 72°C for 5 min, performed in 100-μl reaction mixtures by using the buffer supplied with the enzyme, 200 μM (each) deoxynucleoside triphosphates, 2 mM $MgSO_4$, 1 μg of plasmid DNA, 50 pmol of each primer, and 2 U of Vent DNA polymerase (New England Biolabs, Inc.). The PCR products were gel purified and self-ligated. After transformation, the plasmids prepared were examined for the presence of the restriction site expected to be generated by the deletions. Two deletions were constructed within the region of the IR: the one, using primers IRF1 and IRR1, resulted in plasmid pGS-AG-16-D1, and the second, using primers IRF1 and IRR2, resulted in plasmid pGS-AG-16-D3. In both plasmids, we expected the generation of a *Bam*HI site if the PCR product was ligated correctly. Three deletions were constructed in the promoter region: the one using primers UPR1 and UPF2 resulted in plasmid pGS-AG-16-U2, the one using primers UPR1 and UPF3 resulted in plasmid pGS-AG-16-U3, and the third, using primers UPR1 and UPF4, resulted in plasmid pGS-AG-16-U4. In these plasmids, an *Eco*RI site was expected to be generated by the deletions. All deletions were confirmed by sequencing. The *Eco*RI-*Sal*I fragment was excised from each of the five pGS-AG-16 derivatives and exchanged with the same fragment of pGS-AG-22-S. The inserts of these plasmids were cloned into pGS-SV-1 by using the *Eco*RI and *Hin*dIII sites, resulting in plasmids pGS-SV-22-D1, pGS-SV-22-D3, pGS-SV-22-U2, pGS-SV-22-U3, and pGS-SV-22-U4. The resulting plasmids were conjugated into *A. tumefaciens* (35).

DNA sequencing. Sequences were determined by the dideoxy chain termination method (32) with a Sequenase II sequencing kit (United States Biochemicals Corp.).

RESULTS

The experimental system used for studying the IR preceding the *groESL* operon. In order to find out if the IR is involved in regulating the heat shock response of the *groESL* operon, two deletions were introduced into it. Because the *groESL* operon was shown to be essential for growth in *E. coli* (12), we analyzed these deletions on a plasmid, without changing the chromosomal operon. In order to distinguish between the chromosomal and plasmid *groESL* operon, a 1.4-kb internal fragment was deleted from the plasmid *groEL* gene to generate the *groESL*∗ operon, which codes for an mRNA of 0.7 kb instead of the original 2.1 kb (the construction of this system has been described in detail before [35]). The smaller *groESL*∗ transcript (0.7 kb) can be distinguished from the chromosomal *groESL* transcript (2.1 kb) on Northern blots. The plasmid pGS-AG-22-S, harboring the *groESL*∗ operon, was introduced into *A. tumefaciens*, and after it was confirmed that the 1.4-kb deletion had no effect on heat shock activation of this operon, the deletions generated were transferred to it. The deletions constructed within the IR region are shown in the upper part of Fig. 1.

The *groES* gene was used as a probe for all experiments; it hybridizes with the chromosomal 2.1-kb *groESL* mRNA and with the plasmid 0.7-kb *groESL*∗ mRNA, both of which contain a complete *groES* gene. We did not use a fragment of the *groEL* gene as a probe because as we have shown previously (35), the *groESL* transcript is cleaved between *groES* and *groEL*, separating the *groEL* transcript from the *groES* transcript containing the IR.

Transcriptional analysis of deletions within the IR. In deletion D3, one side of the IR (11 bases) was deleted (Fig. 1). The results of Northern hybridization of RNAs prepared before and after heat shock activation (5 and 10 min) from cells containing plasmids pGS-SV-22-D3 and pGS-SV-22-S (wild-type IR) are presented in Fig. 2A. The two far right lanes show the mRNA levels before heat shock to focus on the major differences between the mRNAs generated from these two plasmids. Deletion D3 resulted in a higher level of mRNA, in comparison to that of wild-type IR, and in reduced heat shock activation. These results are quantitated in Table 2.

A different result was obtained when we analyzed deletion D1 (Fig. 1), in which 24 bases, representing most of the IR, were deleted. Deletion D1 is 4 bases from the transcription start site. We did not construct a complete deletion of the IR because it could affect the transcription of the operon. As can be seen in Fig. 2B, the levels of the mRNA of the operon containing deletion D1 were very low in comparison to those of the operon containing the wild-type IR. We tested this deletion to see if it had any effect on the stability of the plasmid or on the expression of other genes located on the vector, but no such effects were observed (data not shown). In order to compare the magnitude of heat shock activation of the D1 deletion with that of the wild-type IR, 1/10 (1 μg) of the RNA prepared from cells containing the wild-type IR was applied on the gel (Fig. 2B). Deletion D1 had a lower magnitude of activation than did the clone containing the wild-type IR (Table 2), but heat shock activation was observed.

The effect of the deletion within the IR on mRNA half-life. The conserved IR in the *groESL* operon of *A. tumefaciens* (34) and in many other *dnaK* and *groE* operons constitutes part of the transcript (11, 20, 27, 28, 33, 42, 46). In order to study the role of the IR at the mRNA level, we analyzed the effect of deletion D3 on mRNA half-life (deletion D1 could not be examined because it has a very low mRNA level). The mRNA half-life was determined under heat shock and non-heat shock

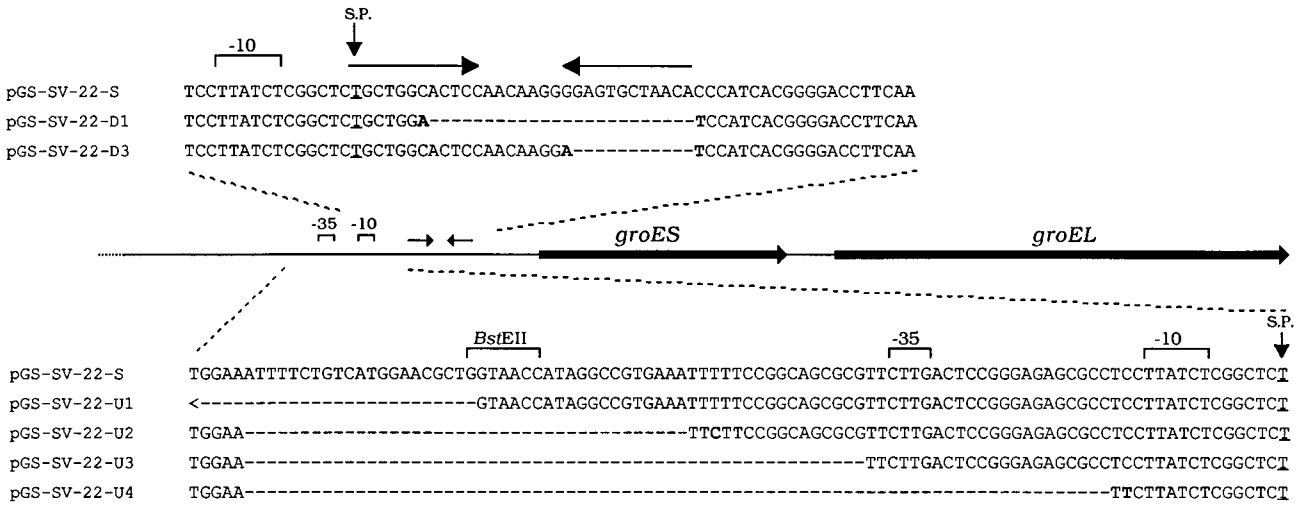

FIG. 1. Deletions constructed in the *groESL* operon regulatory region. In the upper part, deletions constructed within the IR are presented. In the lower part, deletions constructed in the promoter upstream region are shown. The bases that were changed are in boldface; dashes indicate the bases that were deleted. The expected promoter regions are marked −10 and −35; the transcription start site is marked S.P.; the *Bst*EII site used for the construction of deletion U1 is marked.

conditions. Under heat shock conditions (42°C), no difference in the mRNA half-life was observed between clones containing this deletion and the wild-type IR; it was calculated to be approximately 5 min (data not shown). However, under non-heat shock conditions (25°C), large differences were observed.

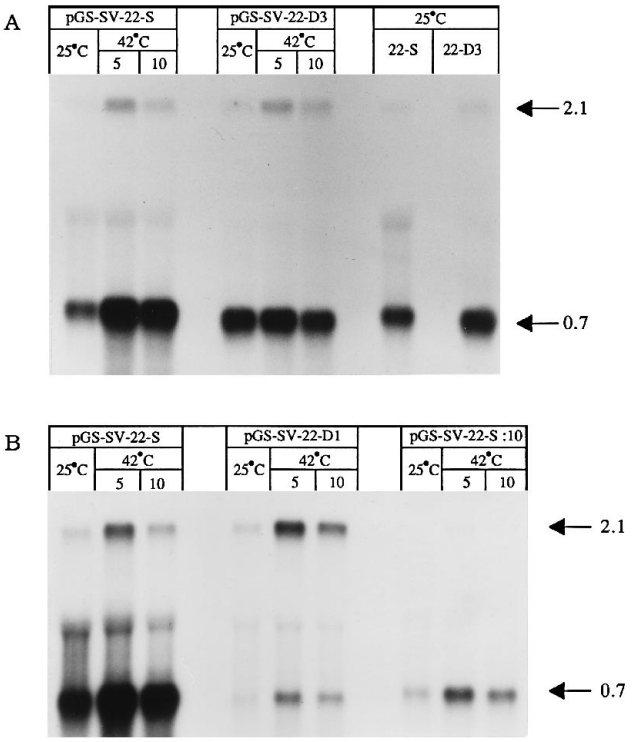

FIG. 2. Northern hybridization of RNAs from *A. tumefaciens* cells containing plasmids pGS-SV-22-S and pGS-SV-22-D3 (A) or pGS-SV-22-S and pGS-SV-22-D1 (B). The two far right lanes in panel A present side by side the levels of transcripts from these two clones at 25°C. Each of the three far right lanes in panel B contained 1 μg of RNA instead of 10 μg. Total RNA was isolated from *A. tumefaciens* before heat shock (25°C) and at different times (5 and 10 min) after heat shock to 42°C. Hybridization was done with the *groES* probe. The positions of *groESL* (2.1-kb) and *groESL** (0.7-kb) transcripts are shown on the right.

The mRNA containing the wild-type IR had a half-life of approximately 12 min, while the half-life of the transcript containing deletion D3 was 20 min (Fig. 3). Similar results were obtained in an analysis of two point mutations within the IR (unpublished results).

Transcriptional analysis of the *A. tumefaciens groESL* operon in *E. coli*. In order to further characterize regulation of the heat shock response of *A. tumefaciens*, we examined heat shock activation of the *A. tumefaciens groESL* operon in *E. coli*. The heat shock response in *E. coli* is known to be mediated by sigma-32, a homolog of which was found lately in *A. tumefaciens* (26). *E. coli* does not contain the IR in the *groE*, *dnaK*, or any other gene. In addition, the *E. coli* heat shock consensus promoter sequence is not homologous to that found in the *A. tumefaciens groESL* operon (34).

RNAs were prepared from *E. coli* cells harboring plasmid pGS-AG-3a (34), which contains the complete *A. tumefaciens groESL* operon. The results of primer extension analysis with a primer specific for the *A. tumefaciens groESL* operon (PEA1) are presented in Fig. 4A. The same RNAs were used for primer extension with a primer specific for the *E. coli groESL* operon (PEC1) (Fig. 4B). It can be clearly seen that the *A.*

TABLE 2. Heat shock activation and mRNA levels of deletions within the IR

Plasmid	Magnitude of activation[a]				mRNA level before heat shock (%)[b]	
	Clone		Chromosomal operon		Clone	Chromosome
	5 min	10 min	5 min	10 min		
pGS-SV-22-S	3.1 ± 0.6	2.5 ± 0.6	3.4 ± 0.6	2.3 ± 0.6	100	100
pGS-SV-22-D1	2.3 ± 0.3	1.7 ± 0.3	3.4 ± 0.6	2.1 ± 0.3	10	100
pGS-SV-22-D3	1.1 ± 0.2	0.8 ± 0.2	3.0 ± 0.6	2.3 ± 0.3	150	100

[a] The magnitudes of activation (after 5 and 10 min of heat shock) were calculated from Northern hybridizations by using a Fuji BAS1000 Phosphor Imager. For each clone, the mRNA level before heat shock was set at 1. Data were obtained from at least five independent experiments for each clone.

[b] The mRNA levels before heat shock of the wild-type clone and chromosomal operon were set at 100%, and all others are referred to them.

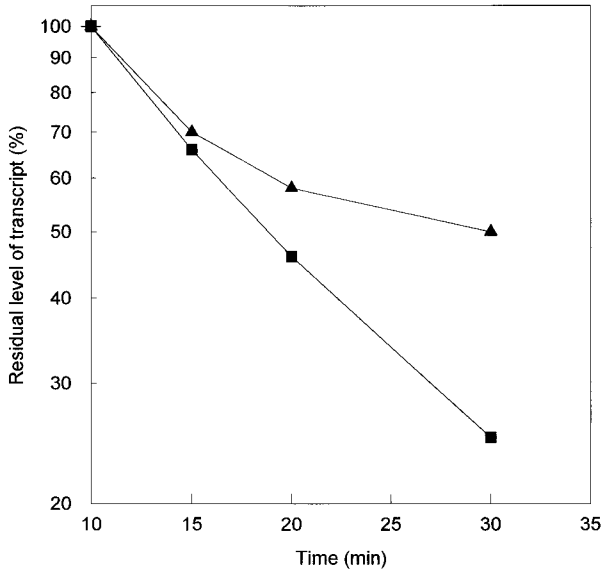

FIG. 3. The stability of transcripts. RNAs were prepared from cells that were growing at 25°C and contained plasmids pGS-SV-22-S (wild-type IR; squares) and pGS-SV-22-D3 (deletion of one side of the IR; triangles). Samples were taken at 10, 15, 20, and 30 min after the addition of rifampin (200 mg/ml). RNAs were analyzed by Northern hybridization, as described in the legend to Fig. 2, and the results were quantitated as described in Materials and Methods. The amount of mRNA at 10 min after the addition of rifampin was set at 100%.

tumefaciens groESL operon was heat shock activated in *E. coli* from the same transcription start site as in *A. tumefaciens* (34). However, the mRNA levels before and after heat shock were very low, although the *A. tumefaciens groESL* operon was cloned on a high-copy-number vector (pUC18).

The reciprocal experiment was also performed; the *E. coli groESL* operon was introduced into *A. tumefaciens*. The insert of plasmid pKT200, which contains the *E. coli groESL* operon (3), was cloned into shuttle vector pGS-SV-1 (35) to generate plasmid pGS-SV-GC. RNAs were prepared from *A. tumefaciens* cells harboring plasmid pGS-SV-GC before and after (5, 10, and 20 min) heat shock. The results of primer extension analysis with primer PEC1 are presented in Fig. 5A, and the results of primer extension of the same RNAs with primer PEA1 are presented in Fig. 5B. The *E. coli groESL* operon was heat shock activated in *A. tumefaciens* from the same transcription start site as in *E. coli*, a transcription start site shown to be recognized by sigma-32 in vitro and in vivo (47). The *E. coli groESL* operon had a high level of expression before heat shock in comparison to that of the *A. tumefaciens groESL* operon, and its level of heat shock activation was lower.

Transcriptional analysis of deletions upstream of the *A. tumefaciens groESL* operon promoter. The results presented so far indicate that the IR and promoter affect the regulation of expression of the *A. tumefaciens groESL* operon. To examine whether there are additional factors in the upstream region of this operon that affect its expression, we constructed four deletions in the upstream region of the *groESL* operon (Fig. 1). In deletion U1, all of the upstream region present in plasmid pGS-SV-22-S (about 1 kb) up to the *Bst*EII site was deleted (the *Bst*EII site is located 73 bases from the transcription start site and 36 bases from the expected −35 region of the promoter). Mutations U2 and U3 were constructed by PCR and contained deletions closer to the −35 region. The results of Northern hybridization of RNAs prepared before and after heat shock activation from cells containing plasmids pGS-SV-22-U1, pGS-SV-22-U2, and pGS-SV-22-U3 are presented in Fig. 6. Deletion U1 did not affect the mRNA level or heat shock activation. The other two mutations resulted in lower levels of mRNA; it was more severe with deletion U3. As the left side of deletion U3 is only 2 bases from the expected −35 region of the promoter, it was expected to affect promoter strength. However, the magnitude of heat shock activation was not changed by deletions U1 and U2, remaining the same as that of the wild-type clone (threefold). Deletion U4 overlaps the expected promoter region, and no *groESL*∗ mRNA was observed from plasmid pGS-SV-22-U4 (data not shown). The results obtained with these deletions suggest that the upstream region of the *groESL* operon does not contain sequences important for heat shock activation of this operon.

DISCUSSION

The results presented here deal with regulatory factors and elements affecting transcription of the *A. tumefaciens groESL* operon under heat shock and non-heat shock conditions. Our results indicate that transcription of the *groESL* operon is controlled by the IR sequence in the upstream region of this operon, which operates at the DNA and mRNA levels. At the DNA level, it probably functions as an operator site that binds a repressor, and at the mRNA level, it determines the half-life of the transcript under non-heat shock conditions. This IR probably operates together with a promoter recognized by a heat shock sigma factor (sigma-32 homolog) that appears to be responsible for transcription of this operon.

The same IR has been shown to be involved in regulation of the heat shock response in the *groE* and *dnaK* operons of *B.*

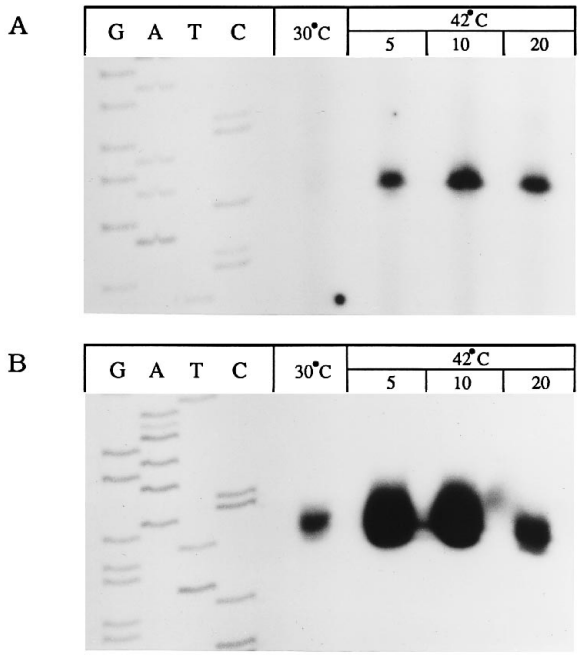

FIG. 4. Primer extension analysis of the *A. tumefaciens groESL* operon in *E. coli*. Total RNA was isolated from *E. coli* cells containing plasmid pGS-AG-3a before heat shock (30°C) and at different times (5, 10, and 20 min) after heat shock to 42°C. (A) Primer extension with the PEA1 primer, complementary to the 5′ end of the *A. tumefaciens groES* gene; (B) primer extension with the PEC1 primer, complementary to the 5′ end of the *E. coli groES* gene. The primer extension products were analyzed on a sequencing gel. Lanes G, A, T, and C, products of the sequencing reaction obtained with the same primer used in primer extension.

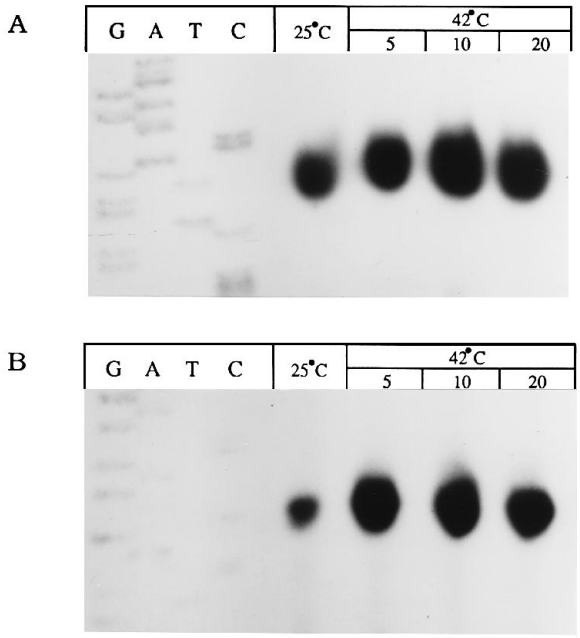

FIG. 5. Primer extension analysis of the *E. coli groESL* operon in *A. tumefaciens*. Total RNA was isolated from *A. tumefaciens* cells containing plasmid pGS-SV-GC before heat shock (25°C) and at different times (5, 10, and 20 min) after heat shock to 42°C. (A) Primer extension with the PEC1 primer, complementary to the 5′ end of the *E. coli groES* gene; (B) primer extension with the PEA1 primer, complementary to the 5′ end of the *A. tumefaciens groES* gene. The primer extension products were analyzed on a sequencing gel. Lanes G, A, T, and C, products of the sequencing reaction obtained with the same primer used in primer extension.

subtilis (46, 49) and in the *dnaJ* gene of *L. lactis* (39). Our deletion of one side of the IR (deletion D3) had effects similar to those observed for the mutations and deletions examined in *B. subtilis* and *L. lactis*, a high mRNA level before heat shock and a reduction in heat shock activation. The high level of the transcript before heat shock is probably due to the loss of repression. In *B. subtilis*, the Orf39 protein encoded by the first gene of the *dnaK* operon was shown to operate as the repressor that binds the IR (45). A homolog of this protein was found in *C. crescentus*, in which it is encoded by the first gene in an operon with the *grpE* gene (GenBank accession no. U33324). Most likely, a homolog of this gene will also be found in *A. tumefaciens*, as both bacteria are alpha purple proteobacteria (29, 43).

The reduction in the magnitude of heat shock activation (Table 2) with deletion D3 is probably due mainly to the longer half-life of its transcript under non-heat shock conditions. The transcript containing deletion D3 had a half-life (20 min) under non-heat shock conditions that was reduced (5 min) in heat shock. In contrast, the mRNA half-life of the transcript containing the wild-type IR was 12 min under non-heat shock conditions and was reduced to 5 min in heat shock. If we assume that deletion D3 did not change the magnitude of activation, the fourfold reduction in the mRNA half-life in comparison to the twofold reduction in the mRNA half-life of the clone containing the wild-type IR can account for most of the reduction in the magnitude of heat shock activation.

Under heat shock conditions, there was no detectable effect of deletion D3 on the half-lives of transcripts. This finding is probably due to the fact that the *groESL* transcript is cleaved during heat shock (35); this cleavage would mask any change in the stability of the *groESL* transcript.

Two previous reports indicated that *dnaK* operons from alpha purple proteobacteria could be heat shock activated in *E. coli*. A *lac* fusion of the *dnaK* operon from *Z. mobilis* was thermoregulated in *E. coli* (24), and the *dnaK* operon of *Brucella ovis* was expressed in a heat shock-dependent manner in *E. coli* (8). These two *dnaK* operons do not contain the IR found in the *groESL* operons of alpha purple proteobacteria. In another study (23), the *E. coli dnaK* gene was introduced into *A. tumefaciens* and was shown to be heat shock activated from the same transcription start site as in *E. coli*.

In this study, we analyzed heat shock activation of the *A. tumefaciens groE* operon in *E. coli* and also performed the reciprocal experiment. The *A. tumefaciens groESL* operon was expressed in *E. coli* in a heat shock-dependent manner, but its mRNA level was very low before and after heat shock (Fig. 4A). On the other hand, the *E. coli groESL* operon had a high mRNA level in *A. tumefaciens* before heat shock and a lower level of heat shock activation (Fig. 5A). These results are compatible with our previous observation that there are differences in the heat shock promoter consensus sequences between alpha and gamma purple proteobacteria (36).

The low-level expression of the *A. tumefaciens groESL* operon in *E. coli* can also be a result of additional factors needed for its expression that are not present in *E. coli*. This possibility was examined by constructing a number of deletions in the upstream region of the *groESL* operon (deletions U1 to U3). The results obtained with these deletions, together with the results obtained with a deletion, D1, within the IR, indicate that the capability for heat shock activation of the *A. tumefaciens groESL* operon is located within the promoter region. We think that the low mRNA levels found with deletions D1 and U3 were a result of the short distance between the promoter or the transcription start site and these deletions. However, in the case of deletion U3, it is also possible that low-level expression was due to removal of the A-track 13 bp upstream of the putative −35 region of the promoter.

Recently, a homolog of the *E. coli* heat shock sigma factor (sigma-32) was cloned from *A. tumefaciens* (26). A comparison of heat shock sigma factors from alpha and gamma purple proteobacteria indicated the existence of several differences. Changes were found in amino acids from regions, 2.4 and 4.2 (Fig. 7B), previously shown to be involved in promoter recognition by different sigma factors (15, 22, 37, 40). These changes

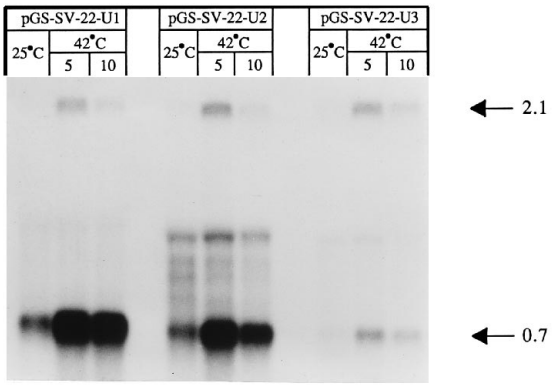

FIG. 6. Northern hybridization of RNAs from *A. tumefaciens* cells containing plasmids pGS-SV-22-U1, pGS-SV-22-U2, and pGS-SV-22-U3. Total RNA was isolated from *A. tumefaciens* cells before heat shock (25°C) and at different times (5 and 10 min) after heat shock to 42°C. Hybridization was done with the *groES* probe. The positions of *groESL* (2.1-kb) and *groESL** (0.7-kb) transcripts are shown on the right.

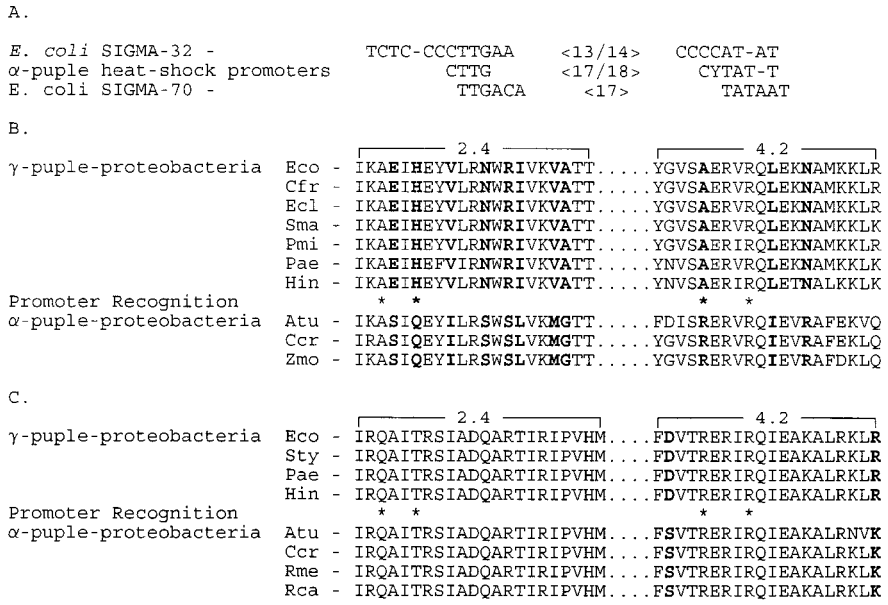

FIG. 7. (A) Comparison of the *E. coli* vegetative sigma factor promoter consensus sequence (*E. coli* sigma-70) (47), the *E. coli* heat shock sigma factor promoter consensus sequence (*E. coli* sigma-32) (47), and the putative heat shock promoter consensus sequence of alpha purple proteobacteria (36); (B) comparison of regions 2.4 and 4.2 from heat shock sigma factors of gamma and alpha purple proteobacteria; (C) comparison of regions 2.4 and 4.2 from vegetative sigma factors of gamma and alpha purple proteobacteria. Bacteria are identified by the following three-letter codes: Atu, *A. tumefaciens*; Ccr, *C. crescentus*; Cfr, *Citrobacter freundii*; Eco, *E. coli*; Ecl, *Enterobacter cloacae*; Hin, *Haemophilus influenzae*; Pae, *Pseudomonas aeruginosa*; Pmi, *Proteus mirabilis*; Rca, *Rhodobacter capsulatus*; Rme, *Rhizobium meliloti*; Sma, *Serratia marcescens*; Sty, *Salmonella typhimurium*; and Zmo, *Z. mobilis*. In panels B and C, amino acids found to be conserved in one evolutionary group but different in the other are in boldface. *, amino acid position known to be involved in promoter recognition. The sequences of the heat shock sigma factors are from the following GenBank accession numbers: Eco, X04398; Cfr, X14960; Ecl, D50829; Sma, D50831; Pmi, D50830; Pae, P42378; Hin, P44404; Atu, D50828; Ccr, U37792; and Zmo, D50832. The sequences of the vegetative sigma factors are from the following GenBank accession numbers: Eco, J01687; Sty, M14427; Pae, D90118; Hin, P43766; Atu, X69388; Ccr, U35138; Rme, L47288; and Rca, P46400.

are found in all of the heat shock sigma factors cloned from alpha purple proteobacteria and also involve differences in charged amino acids (histidine to glutamine and asparagine to arginine). When a similar comparison between vegetative sigma factors from the two divisions was performed, very few changes were found in these two regions and no changes were found in amino acids known to be involved in promoter recognition (Fig. 7C). The changes found between the heat shock sigma factors of these two divisions are probably correlated with changes in the heat shock promoter sequences of these two groups (Fig. 7A).

Regulation of the *groESL* operon of *A. tumefaciens* is complex, since it probably involves an alternative heat shock sigma factor, the IR control element, and posttranscriptional regulation. Under non-heat shock conditions, transcription of this operon is repressed via the IR, which also destabilizes the transcript. We assume that the recently discovered heat shock sigma factor is responsible for the expression of this operon under both heat shock and non-heat shock conditions. Under heat shock conditions, the repression that involves the IR is presumably released and the heat shock sigma factor activates transcription of this operon. The stability of the transcript under these conditions is determined not by the IR but by heat shock-dependent cleavage (35). This heat shock control system involves three regulatory elements, and it is possible that under different stress or growth conditions the relationships among them change, resulting in a level of expression that is suited to the required levels of GroEL and GroES in the cell.

The IR control system found in almost all eubacterial divisions is highly conserved in sequence and in function in the *groESL* operon of *A. tumefaciens* (alpha purple proteobacteria) and in the *groESL* operon of *B. subtilis* (gram-positive bacteria). Its wide distribution, its high-level sequence conservation, its regulation of chaperon-encoding genes, and its complex regulatory functions at both the DNA and mRNA levels indicate that the IR control system is an ancient and highly important regulatory system.

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